INTRODUCTION

The principle of cDNA microarray hybridization takes advantage of the property of DNA to form duplex structures between two complementary strands. In this technique, the cDNA probes, which are arrayed onto a glass slide and represent the sequence of known genes or expressed sequence tags (ESTs), interrogate fluorescently labeled cDNA targets synthesized from extracted mRNA. In two-color microarray experiments, the differentially labeled cDNA targets (e.g from tumor and normal tissue) hybridize to their respective cDNA probe sequences tethered to the slide. After imaging the microarray slide for signal intensities in each color channel, the relative expression ratio for each arrayed gene can be determined. In contrast to traditional gene-by-gene expression monitoring (such as Northerns), the cDNA microarray technique is limited only by the number of genes printed on the slide, and therefore allows for the analysis of gene expression on a truly genome-wide scale. *Note 1*.

Gene expression monitoring using microarrays was described in 1994 by Drmanac *et al.* (1; 2) who used radioactive targets hybridized onto filter-immobilized PCR-amplified cDNA probes. In 1995, Schena *et al.* (3) first described the hybridization of two-color fluorescently labeled targets to cDNA microarrays printed on glass. The two-color detection scheme has the advantage over radioactively labeled targets by allowing rapid and simultaneous differential expression analysis of two biological samples, with one color used as a reference for normalization purposes. The reference allows for compensation of target-to-target and slide-to-slide variations of intensity due to DNA concentrations and hybridization efficiencies, and thereby permits comparisons between multiple biological samples across many experiments.

With over 1,800,000 human expressed sequence tag (EST) sequences available (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html), representing 50-90% human genes, it is now possible to uncover the gene expression profiles of human cancers, querying the expression of thousands of genes in a single experiment. Global gene expression of different types of cancer may allow the development of expression

profiles unique for a cancer (4-7) and may lead to the development of rapid diagnostic assays. It may also identify secreted and membrane proteins (8) that can be used for early diagnosis and for monitoring therapy. Gene expression profiles can also be correlated with clinical data to help predict biological behavior (9), and may allow us to direct therapy. In addition, this information may be useful in dissecting out the pathways involved in therapy failure (10), or malignant transformation with oncogenic transcription factors (11), and may ultimately provide novel therapeutic targets. Interest in microarray technology has risen in the pharmaceutical industry for new cancer drug discovery and for monitoring the effects of novel therapeutic agents (12). The list of potential uses of this technique is endless and is not limited to cancer research (13, 14).

The protocols are meant to be a general guide to setting up a microarray facility; other kits, reagents, and protocols may be substituted where necessary.

MATERIALS

All materials may be stored at room temperature (R/T) unless otherwise noted.

1.1. Clone Production

LB broth (Biofluids Division, BSI, Rockville, MD, #359-000)
Superbroth (Biofluids Division, BSI, Rockville, MD, #371-000)
96-well round bottom plates (Corning Inc, Corning, NY, #3799)
ThinSeal Plate Sealers (Excel Scientific, Wrightwood, CA, #STR-THIN-PLT)
96 well Culture Blocks (Edge BioSystems, Gaithersburg, MD, #4050066)
Airpore Tape Sheets (Qiagen, Valencia, CA, #19571)
Carbenicillin
Ampicillin
96-pin inoculation stamp

100% Ethanol

1.2. Isolation of Plasmid DNA

96-well Alkaline Lysis Miniprep Kit (Edge BioSystems, Gaithersburg, MD, #91528)
[store all buffers at 4°C]
1M Tris-HCl pH 8.0 (Gibco-BRL, Rockville, MD, #5568UA)
0.5M EDTA pH 8.0 (Research Genetics, Huntsville, AL, #750009)
100% Ethanol

1.3. PCR Amplification of Clones

Cycleplate Thin wall PCR plate (Robbins Scientific, Sunnyvale, CA, #1038-00-0)
Cycleseal PCR Plate Sealer (Robbins Scientific, Sunnyvale, CA, #1038-00-0)
We use MJ Research (DNA Engine Tetrad) PTC-225 Peltier Thermal Cyclers
10X PCR Buffer (PerkinElmer, Wellesley, MA, #N808-0189) [4°C]
Ampli Taq Polymerase (PerkinElmer, Wellesley, MA, #N808-4015) [-20°C]
dNTPs (100mM stocks) (Amersham Pharmacia, Piscataway, NJ, #27-2035-02) [-20°C]
DEPC H2O (Research Genetics, Huntsville, AL, #750024)
AEK M13 forward (F) and reverse (R) primers, a custom oligo (Midland Certified, Midland, TX) [-20°C]:

AEK M13F 5'-GTTGTAAAACGACGGCCAGTG-3' stock concentration 1 mM.
AEK M13R 5'-CACACAGGAAACAGCTATG-3' stock concentration 1 mM.

1.4. Quantification of PCR Product

FluoReporter Blue Fluorometric dsDNA Kit (Molecular Probes, Eugene, OR, #F-2962) [4°C]

Microfluor 2 White 96-well U-bottom plates (Dynex, Chantilly, VA, #7105)

Lambda HindIII fragments (Gibco-BRL, Rockville, MD, #15612-013) [-20°C]

PerkinElmer Luminescence Spectrometer LS50B

1.5. PCR Product Purification

96-well V-bottom plates (Corning Inc, Corning, NY, #3894)

Cyclefoil plate sealers (Robbins Scientific, Sunnyvale, CA, #1044-39-3)

Super T21 Centrifuge (Sorvall, Newtown, CT)

1575 ImmunoWash (Bio-Rad, Hercules, CA)

100% Ethanol, 70% Ethanol

3M Sodium acetate buffered to pH 6.0

3X SSC

Quart size heat sealable bags and (Kapak, Minneapolis, MN, #404)

Electric sealer

1.6. Poly-L-lysine Pre-treatment of Glass Slides

Gold Seal slides (these slides have consistently low intrinsic fluorescence) (Becton Dickinson, Franklin Lakes, NJ , #3011)

50-slide Stainless steel slide racks and glass tanks (Wheaton, Millville, NJ, #900400)

Sodium hydroxide (pellets)

100% Ethanol (the source alcohol should be examined in a fluorometer to insure that it has very low levels of contaminating fluorescent organic compounds)

0.1% w/v Poly-L-lysine (Sigma, St. Louis, MO, #P8920)

Tissue Culture PBS (Sodium chloride 8g/L, Potassium chloride 0.2g/L, Sodium phosphate dibasic anhydrous 1.44g/L, Potassium phosphate monobasic 0.24g/L) [sterilized and filtered]

25-slide plastic slide racks and plastic tanks with lids (Shandon Lipshaw, Pittsburgh, PA, #195 and #196)

1.8. Blocking Slides after Printing with Succinic Anhydride

1-Methyl-2-pyrrolidinone (Aldrich, Milwaukee, WI, #32,863-4)

Succinic anhydride (Aldrich, Milwaukee, WI, #23,969-0)

1M Sodium borate pH 8.0 (adjust pH of boric acid with sodium hydroxide)

Stratagene UV Stratalinker 2400

30-slide stainless steel slide rack (Wheaton, Millville, NJ, #900234)

30-slide glass submersion tanks (Wheaton, Millville, NJ, #900303)

100% Ethanol

500 ml glass beakers and stir bars

Large glass dish (14 inch casserole)

Large round Pyrex dishes (8 inch diameter)

Plastic slide box

2.1. RNA Extraction

Virsonic 100 with micro probe (conical titanium probe, 1.8 mm diameter tip) (Virtis, Gardiner, NY, #346411)

RNeasy Midi Kit (Qiagen, Valencia, CA, #75142)

Trizol (Gibco-BRL, Rockville, MD, #15596-018) [4°C]

Microcon-30 (Millipore, Bedford, MA, #142410)

Beta-mercaptoethanol (ICN Biomedicals, Aurora, OH, #806445)

DEPC H2O (Research Genetics, Huntsville, AL, #750024)

Chloroform

Isopropanol

50 mM Sodium hydroxide

70% Ethanol

2.2. Direct Labeling of cDNA with Fluorescent Dyes

10X low T dNTPs nucleotide mix [-20°C]:

dNTP	µl	mM final (1/10) concentration
dGTP	25	0.5
dATP	25	0.5
dCTP	25	0.5
dTTP	10	0.2
DEPT H2O	415	-
Total Volume	500	

(100mM dNTPs from Amersham Pharmacia, Piscataway, NJ, #27-2035-02)

FluoroLink Cy3-dUTP 1 mM (Amersham Pharmacia, Piscataway, NJ, #PA53022) [photosensitive][-20°C]

FluoroLink Cy5-dUTP 1 mM (Amersham Pharmacia, Piscataway, NJ, #PA55022) [photosensitive][-20°C]

(Cye dyes are also available from NEN Life Sciences, Boston, MA)

SuperScript II reverse transcriptase enzyme, 5X 1st Strand Buffer, 0.1M DTT (Gibco-BRL, Rockville, MD, #18064-014) [-20°C]

Anchored oligo-dT (d-20T-d(AGC)) (1 µg/µl) primer [-20°C]

Rnase Inhibitor RNAsin (Promega, Madison, WI, #N211A) [-20°C]

DEPC H2O (Research Genetics, Huntsville, AL, #750024)

2.3. Target Purification

0.5M EDTA pH 8.0 (Research Genetics, Huntsville, AL, #750009)

1M Tris-HCl pH 7.5 (Quality Biological, Gaithersburg, MD, #351-006-100)

1M Sodium hydroxide

3.1. Microarray Assembly

Poly dA (10mg/ml) (Sigma, St. Louis, MO, #P9403) [-20°C]
Yeast tRNA (4mg/ml) (Gibco-BRL, Rockville, MD, #15401-011) [-20°C]
Human Cot-1 DNA (concentrated to 10mg/ml) (Gibco-BRL, Rockville, MD, #15279-011) [-20°C]
50X Denhardts (Research Genetics, Huntsville, AL, #750018) [-20°C]
10% SDS (Research Genetics, Huntsville, AL, #750008)
Slide hybridization chamber (TeleChem, Sunnyvale, CA, #AHC-1)
20X SSC

3.2. Post-Hybridization Slide Washes

Glass coplin staining jars (Wheaton, Millville, NJ, #900470)
25-slide plastic slide racks (Shandon Lipshaw, Pittsburgh, PA, #195)
Wash Solution #1: 0.1% SDS + 0.5 X SSC in ddH2O [filtered]
Wash Solution #2: 0.01% SDS + 0.5 X SSC in ddH2O [filtered]
Wash Solution #3: 0.06X SSC in ddH2O [filtered]

METHODS

1. cDNA Microarray Production.

The choice of which genes or ESTs to print are user specified. For human cancer profiling, we are currently using the "22K" human gene set comprised of 22,320 human UniGene clones available from the IMAGE consortium and distributed by Research Genetics (Huntsville, AL). Within the set are approximately 4,000 clones corresponding to known genes; the rest are unknown genes or ESTs. Currently, arrays of up to 15,000

probes are printed. The probe DNA is made from the IMAGE clones, which are arrayed in 96-well format and are used as template for PCR amplification.

Pre-fabricated high-density microarrays can be currently purchased from a number of sources such as TeleChem (http://www.arrayit.com). Companies such as Incyte (http://www.incyte.com/) offer microarray hybridization and analysis services to investigators who provide the RNA. The Affymetrix GeneChips (http://www.affymetrix.com) are high-density arrays of oligonucleotide probes synthesized simultaneously on a large glass wafer by photolithography. Protocols for RNA preparation and microarray hybridization for these commercial solutions will vary considerably.

1.1. Clone Production

Methods:

Pre-growth of clones to ensure maximum plasmid production. *Note 2.*

a) In sterile 96-well round bottom plates, add 100 µl LB broth per well with 100 µg/ml carbenicillin.
b) Thaw frozen 96-well library plates containing source bacterial cultures and spin briefly, two minutes at 1000 rpm, to remove condensation and droplets from the sealer.
c) Sterilize the 96-pin inoculation stamp between samples using 100% ethanol and flame the pins using appropriate safety precautions.
d) After briefly allowing the inoculation block to cool, dip the pins in the library plate, then inoculate the equivalent LB plate ensuring correct orientation. Sterilize the inoculation pins as in (c) above.
e) Reseal the library plates with plate sealers (ThinSeal). Store the library plates at –70°C.

f) Incubate growth plates in humidified oven overnight at 37°C.

Inoculating deep well culture blocks.

a) Add 1ml of Superbroth, containing 100 µg/ml carbenicillin, to each well of the 96-well culture blocks using an eight channel pipetter.
b) Using the 96-pin inoculation stamp, as above, inoculate the 96-well culture blocks.
c) Cover (Airpore sheets) and place blocks in the 37°C shaker incubator (200 rpm) for 24 hours.

1.2. Isolation of Plasmid DNA.

Methods:

a) Isolate plasmid DNA from the cultures using the miniprep kit according to the manufacturer's protocol.
b) Resuspend the plasmid DNA in 200 µl T. low E. (10mM Tris-HCl, 0.1mM EDTA).
c) The DNA is then stored at –20°C and used as template for PCR amplification (see protocol 1.3).

1.3. PCR Amplification of Clones.

The isolated DNA is used as a template for PCR amplification with vector primers (AEK-M13) using a 96-well format, typically 12 plates at a time.

Methods:

a) A PCR reaction mix is made by combining the following on ice:

Reagent	[Stock]	[Final]	Volume per 1000 Reactions

PCR buffer	10X	1X	10 ml
dATP	100 mM	0.2 mM	0.2 ml
dTTP	100 mM	0.2 mM	0.2 ml
dGTP	100 mM	0.2 mM	0.2 ml
dCTP	100 mM	0.2 mM	0.2 ml
AEK M13F	1000 µM	0.5 µM	0.05 ml
AEK M13R	1000 µM	0.5 µM	0.05 ml
Ampli Taq Pol.	5 U/µl	0.05 U/µl	1 ml
DEPC H2O			87.1ml

Note 3.

b) Using a multichannel pipette, transfer 99 µl of the master mix to each well of PCR plate (Cycleplate).

c) Using a multichannel pipette, transfer 1 µl of appropriate template DNA in each well taking care to keep the plate orientation and order. *Note 4.*

d) Cover the plates with sealers (Cycleseal) and place in thermocycling device.

e) Amplify the templates using the following cycle conditions:

Step	Temperature	Time
1.	96°C	30 sec.
2.	94°C	30 sec.
3.	55°C	30 sec.
4.	72°C	150 sec.
5.	Repeat steps 2 to 4, 24 times	
6.	72°C	5 min

1.4. Quantification of PCR Product.

Methods:

a) Two µl of each PCR product is analyzed by electrophoresis on a 2% TAE agarose gel containing 0.5 µg/ml ethidium bromide. We obtain a digital image of the gel under UV illumination and analyze the electrophoresis products to ensure that a single band

of distinct size is produced for each sample. The intensity of the band gives an estimate of the relative amount of product.

b) Quantification of PCR products can be accomplished using flurometric quantitation. *Note 5.*

c) Expected yield is ~100µg/ml.

1.5. PCR Product Purification.

Methods:

a) Prepare an ethanol/acetate precipitation mix (150 mM sodium acetate pH 6 in ethanol). Add 200 µl of the precipitation mix to each well of a V-bottom 96-well plate.

b) Using multi-channel pipetter, transfer the remaining (approx. 97 µl) PCR products to their corresponding wells containing the precipitation mix. *Note 6.*

c) Place plates in the -80°C for a period of one hour, or overnight at –20°C, to precipitate the DNA.

d) Allow plates to thaw (to reduce brittleness and melt any ice) and spin in a high-speed swinging holder centrifuge (Sorvall Super T21). We typically spin stacks of 3 plates at 1600 rcf for 1 hour. *Note 7.*

e) After centrifugation, remove supernatant from plates and dispense 70% ethanol wash, 150 µl per well using a plate processing station such as the Bio-Rad 1575 ImmunoWash.

f) Centrifuge the plates as in step (d) at 1600 rcf for 1 hour, and remove the supernatant. In a dust-free area, allow plates to dry overnight, without lids and covered with clean paper towels.

g) Resuspend the PCR products in 40 µl of 3X SSC. Seal plates with foil sealer, making sure that all wells are tightly sealed. Place the plates in an air-tight heat sealed bag with a moistened paper towel and place in a 65°C oven for 2 hours. *Note 8.*

h) Remove the cooled plates and store at -20°C.

1.6. Poly-L-lysine Pre-Treatment of Glass Slides.

Treatment of slides with a coat of poly-L-lysine allows the target DNA to adhere to the surface and minimize loss during hybridization. *Note 9.*

Methods:

a) Place new Gold Seal microscope slides into a stainless steel 50-slide rack.
b) Prepare cleaning solution in large glass beaker (500 ml required per 50-slide glass tank):

    400 ml ddH2O
    100g NaOH
    600 ml 95% ethanol

    Dissolve NaOH in water, then add ethanol. Stir until solution is clear. If the solution doesn't clear, add H2O until it does.
c) Dispense cleaning solution into 50-slide glass tanks. Submerge the rack in the cleaning solution and shake for 2 hours on orbital shaker.
d) Remove slides and rinse with fresh ddH2O 2-5 minutes. Repeat wash 4X, each time using fresh ddH2O. *Note 10.*
e) Move clean slides to 25-slide plastic racks.
f) Prepare poly-L-lysine solution as follows (for 2 boxes of 25 slides each):

    35 ml poly-L-lysine (0.1% w/v)
    35 ml Tissue Culture PBS
    280 ml ddH2O
g) Dispense poly-L-lysine solution to plastic 25-slide containers. Submerge rack in poly-L-lysine solution, cover with lid, and shake for 1 hour.
h) Rinse once in ddH2O for 1 minute.
i) Centrifuge rack in a low speed swinging holder centrifuge to remove free liquid.
j) Immediately transfer to a clean slide box.
k) Allow slides to age for 2 weeks before printing. *Note 11.*

1.7. Microarray Slide Printing.

The next stage is printing of DNA probes on the coated glass slides. The printing process refers to the robot-driven sequential transfer of individual purified PCR amplified fragments from a 96-well microtiter tray to exact, predefined locations on glass slides. Several arrayers are available from commercial companies: 1. Affymetrix 417 Arrayer (http://www.affymetrix.com/products/spotted.html); 2. Cartesian Technologies (http://www.cartesiantech.com/); 3. Beecher Instruments (http://www.beecherinstruments.com/); 4. Genomic Solutions (http://www.genomicsolutions.com/); 5. BioRobotics (http://www.biorobotics.co.uk/). It is also possible to build you own arrayer for approximately US $25,000 (http://cmgm.stanford.edu/pbrown/), and arrayers have been built in several academic settings (15).

The Cancer Genetics Branch custom-built arrayers, using "quill" type pens, print sequentially up to 16 spots at once on each of 48 or 96 slides, wash and dry the print pens before picking up the next set of cDNAs, and repeats until a complete 96-well plate of probe DNA has been printed (13). At this time, 96-well plates must be manually changed, however an autoloading mechanism is in development. Each pen collects approximately 200-500 nL and deposits between 2-3 nl (0.2-0.5 ng) of PCR product.

After printing is complete, etch identifying marks along the top of each slide (print number and slide number) with a diamond scriber and place slides in plastic slide box (use simple plastic slide boxes with no paper or cork to shed particles). *Note 12.*

1.8. Blocking Slides after Printing with Succinic Anhydride.

To reduce non-specific binding of strongly negatively charged target on microarray slides, the positively charged amine groups on poly-L-lysine coated slides are passivated by reaction with succinic anhydride. We routinely process 48 slides at a time. *Note 13.*

Methods:

a) Age slides for one week at R/T after printing. *Note 14.*
b) Place slides in glass casserole dish and cover with plastic wrap. UV crosslink printed cDNA with a dose of 450 mJ UV energy (Strategene Stratalinker).
c) Place slides in stainless steel 30-slide racks, and place racks in clean glass tanks. Prepare passivation reaction (for 1 tank) in dedicated, dry, 500 ml beaker:

Succinic anhydride	6 g
1-methyl-2-pyrrolidinone	325 ml
1M sodium borate pH 6.0	25 ml

When succinic anhydride has completely dissolved, add 25 ml of 1M sodium borate buffer while mixing and quickly pour onto slides. *Note 15.*
d) Shake the slides for 20-30 minutes on orbital shaker – some precipitation will occur. While blocking, boil ddH2O in a clean Pyrex dish using a hot plate, so that it will be ready after the reaction. *Note 16.*
e) Remove slide holder from the passivation reaction and dunk immediately in boiling ddH2O to denature DNA. Turn off heat source and let stand for two minutes in the nearly boiling ddH2O bath. Remove slide holder and dunk in fresh glass tank with 100% ethanol to dehydrate slides.
f) After 3-5 minutes in ethanol, remove the slides and centrifuge dry in a low-speed swinging holder centrifuge.
g) Place dry slides in a clean slide box.

2. RNA Extraction and Target Production.

RNAs isolated from the cells or tissue one wishes to analyze are used as the template for synthesis of fluorescently-labeled cDNA targets. For cell lines, RNA first extracted using a Qiagen RNeasy kit followed by a further round of purification using Trizol yields excellent results (two rounds of Trizol extraction is recommended for tissues). The amount of total RNA in each channel required for a microarray experiment varies from 50 to 200 µg, with the precise amount varying with the size of the array and fluorescent nucleotide used. *Note 17.*

Considerable thought should be given to what reference cell line or tissue to use for your microarray experiments. The reference should be abundant and offer at least a minimal intensity for all genes printed on your array (if a gene has no intensity in the reference channel, then ratios and other statistical calculations cannot be computed as the denominator cannot be zero).

2.1. RNA Extraction.

Methods:

a) For the RNeasy kit follow the manufacturer's guidelines. At the final elution stage, elute with two successive aliquots of 150 µl RNase-free water. *Note 18.*
b) Determine the concentration of your RNA in 50 mM NaOH.
c) At this time it may be convenient to aliquot out appropriate quantities (55-220 µg) of your RNA before beginning the second round of purification. *Note 19.*
d) Extract the eluted RNA a second time by adding 1 ml of Trizol per 0.3 ml of eluant, vortexing, and follow manufacturer guidelines for RNA extraction.
e) You may leave the precipitated RNA in isopropanol at -80°C for later use.

f) If the RNA is to be used immediately, wash the RNA pellet 2X with 70% ethanol, remove the ethanol and dry the pellet (air dry or speedvac). *Note 20.*

g) Resuspend the dried RNA pellet (50-200 µg) in 400 µl RNase-free H2O. Take 1 µl for final RNA concentration measurement, and make sure the total amount of RNA for labeling with Cy3 is 80~100µg, and 150~200µg for Cy5.

h) Transfer RNA to Microcon-30 and centrifuge at 14,000 rcf for 7-12 minutes to concentrate RNA. Concentrate RNA to less than 14 µl. *Note 21.*

i) Elute RNA, and bring to a final volume of 14 µl with DEPC H2O.

2.2. Direct labeling of cDNA using Fluorescent Dyes.

The labeling of complex probes is accomplished by direct incorporation of fluorescent nucleotides during a reverse transcription (RT) reaction. Currently the factors of labeling efficiency, fluorescent yield, spectral separation, and non-specific binding make the Cy3/Cy5 pair the most useful for our detection system. Although there are a number of conjugated fluorophores available (dCTP, dUTP, amino-allyl dUTP RT coupled to monofunctional dyes), we have found that Amersham Pharmacia dUTP-conjugated Cy3/Cy5 yields consistent results. Other labeling systems are being tested.

Methods:

a) Pre-anneal RNA with Anchored oligo-dT (d-20T-d(AGC)) (1 µg/µl) primer:

RNA	14 µl
Anchored Oligo-dT primer	3 µl

Incubate in thermocycler 70°C for 5 minutes and cool to 42 °C.

b) Mix the RT labeling reaction:

Cy3 or Cy5-dUTP (1mM)	4 µl
5X First Strand Buffer	8 µl
10X low T dNTP mix	4 µl
0.1M DTT	4 µl

Rnase Inhibitor RNAsin	1 µl
SSII Reverse Transcriptase	2 µl

c) Add RT labeling mix to the pre-annealed RNA.
d) Incubate 42 °C for 30-60 minutes.
e) Add 2 µl SII RT enzyme incubate 42 °C for another 30-60 minutes and cool to R/T.

2.3. Target Purification.

The labeled target reaction must be purified to remove unincorporated nucleotides.

Methods:

a) To stop the labeling reaction, add 5 µl of 0.5M EDTA (pH 8.0) and mix well.
b) To hydrolyze the RNA, add 10 µl 1M sodium hydroxide and mix well. Incubate at 65°C for 20-30 minutes, then cool to R/T.
c) Add 25 µl 1M Tris-HCl (pH 7.5) to neutralize the NaOH.
d) Purify each labeled color individually for the first purification. In Microcon-30 spin columns, add labeled target and bring up to a total volume of 400 µl with DEPC H2O. Spin column at 16,000 rcf for about 8-9 minutes to a volume of approximately 50 µl. *Note 22.*
e) Recover each target. For the second purification, pool the Cy3 and Cy5-labeled targets for an experiment in a new Microcon-30 column and bring up to a total volume of 400 µl H2O.
f) Concentrate the combined targets to 25 µl final volume for hybridization.

3. Hybridization.

Hybridization volumes may vary depending on array size. The following is based on a 20mm by 40mm array. Adjust volumes proportionally and use appropriate sized coverslips for smaller/larger arrays.

3.1. Microarray Assembly.

Methods:

a) Make hybridization mixture containing competitor DNA (to reduce non-specific binding and background):

Pooled Cy5/Cy3 labeled targets	25 µl
Poly dA (10mg/ml)	1.5 µl
Yeast tRNA (4mg/ml)	1.5 µl
Human Cot-1 DNA (10mg/ml)	1.5 µl
50X Denhardts	1.5 µl
20X SSC	5 µl

Note 23.

b) Denature at 98°C for 2 minutes and cool on wet ice for 10 seconds.

c) Add 10% SDS 0.8 µl

d) Pipette hybridization targets up and down several times until well mixed and place mixture on microarray under a 24mm by 50mm glass coverslip. *Note 24.*

e) Place microarray slide in hybridization chamber with 15-20 µl of 3X SSC to maintain humidity within the chamber.

f) Incubate microarray hybridization chamber in 65°C water bath for 12-18 hours.

3.2. Post-Hybridization Slide Washes.

After hybridization, the hybridization solution and any unbound target must be removed from the surface of the slide to reduce background.

Methods:

a) Remove microarray hybridization chamber from water bath. *Note 25.*

b) Dispense wash solutions into coplin staining jars. Open hybridization chamber and immediately place slide in Wash #1 until coverslip slips off. Once coverslip comes off, agitate gently for 2 minutes. *Note 26.*
c) Transfer slide to Wash #2 and agitate gently for 2 minutes.
d) Transfer slide to Wash #3 and agitate gently for 2 minutes.
e) Place the slide in a plastic 25-slide rack and spin in a centrifuge equipped with a swinging carrier (horizontal) which can hold the slide holder. Spin immediately (900 rcf for 2 minutes at R/T).
f) Scan slide as soon as possible.

4. Image Acquisition.

Target fluorescence intensities at the immobilized probes can be measured using a variety of commercially available scanners (16). The following is a brief list of commercial available scanners and contact information.

Affymetrix: 418 Array Scanner – Scanning laser digital imaging epi-fluorescence microscope. 532 nm (35 mW) and 635 nm (35 mW) lasers with 3 minute scan time per slide. Cost: US $50,000. (http://www.affymetrix.com/)

Agilent: under development. (http://www.agilent.com/)

Axon: GenePix 4000 – 532 nm (20 mW) and 635 nm (15 mW) lasers and 10 µm pixel resolution. 5 minute scan time per slide. Cost: US $50,000. (http://www.axon.com/)

Beecher Instruments: Scanner – laser confocal, 2 simultaneous PMT channels. 3 lasers: 488 nm @ 75 mw, 532 nm @ 100 mw , 633 nm @ 35 mw. 10-100 µm pixel resolution. (http://www.beecherinstruments.com/)

Genomic Solutions: GeneTAC LS IV and GeneTAC 2000 – CCD camera with high energy Xenon light source. Can scan up to 4 fluors per slide. Cost: US $60,000. (http://www.genomicsolutions.com/)

GSI Lumonics: ScanArray LITE, ScanArray 4000, ScanArray 5000 – Scanning confocal laser GHeNe 543nm (Cy3) and RHeNe 632nm (Cy5). (http://www.genscan.com/)

Molecular Dynamics: Array Scanner – Confocal optics, 9 element lens. HeNe and NdYag lasers. Scanning time per slide: 5 min single color, 11 min two color. Cost: US $110,000. (http://www.moleculardynamics.com/)

Packard Instruments: BioChip Imager – Epi-fluorescence confocal scanning laser system. 543 nm (Cy3) and 633 nm (Cy5) HeNe lasers with 50 µm, 20 µm, or 10 µm pixel resolution. (http://packardinst.com/)

The scanners used by our lab are Beecher Instruments or Agilent custom-built dual laser confocal microscopes that generate two-color simultaneous digital scans saved to an IBM PC. Intensity data is integrated in 10-15 µm square pixels and recorded at 16 bits.

5. Image Analysis and Normalization.

The two image files generated by the scanner are analyzed using software tools (Array Suite) developed by Chen *et al.* for the ScanAlytics IPLab image processing package (17). These software tools can be used with any image file format to extract raw target intensity information as well as compute background-corrected intensities and expression ratios, confidence intervals, and allow for data integration of all clone information. As each probe is roboticly printed to a pre-defined position, the scanned images are overlaid with a grid that divides the images into segments, each containing a target spot. All clone information, including gene name, clone identification number, chromosome and radiation hybrid mapped location, and source microplate position, is attached to each

segment by this process. Each of the images are assigned a pseudo-color (e.g. Cy5 = red and Cy3 = green). The probe spot is identified within each segment and the target fluorescent intensity is calculated for each color by averaging the intensities of every pixel inside the detected spot region. The local background intensity around each spot in each color is also measured within each segment. For every spot in each color channel, the final target intensity values are derived by subtracting the local background intensity from the average fluorescent intensity.

Next, a normalization constant is determined to compensate for differential efficiencies of labeling and detection of Cy3 and Cy5. The process involves calculating the average intensity, in both color channels, for a set of internal controls consisting of 88 housekeeping genes. These genes are pre-selected and have been verified on numerous hybridizations as being stable for most experiments (red/green ratio = 1.0). The normalization constant is then derived and used to calculate a calibrated red/green ratio for each cDNA spot within the image. In addition the ratio variance of the 88 control genes is used to calculate 99% confidence intervals in which the ratios are statistically no different from 1. The output of the analysis is in the form of a pseudo-colored image of the entire array. Individual spots can be highlighted using the mouse cursor and information including gene name, clone identity, intensity values, intensity ratios, normalization constant and user-defined confidence intervals can be obtained. A spreadsheet of expression ratio data for each spot is generated. For more information about this process and to download free software and tools, see our web site (http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/).

5.1. Sensitivity and Specificity.

It is estimated that that the sensitivity of this method allows the detection of mRNA species comprising 1:10,000 of the mass of poly (A)+. Comparisons between the microarray experiments with Northern hybridizations have confirmed this technique to be reliable (11; 13; 14; 18). Our experience to date has indicated the high consistency of

microarray data for determining ratio changes, however, there is some variation in the exact value of the ratios obtained by these two methods. In some instances the ratio obtained by microarray analysis underestimates that obtained by Northern analysis. Possible causes for this underestimation include reaching a probe intensity saturation limit at the highest intensities under our current detection system. Additionally, the largest ratio changes frequently have one of the measurements near the lower limit of detection, and at these levels the effects of background and non-specific binding are more apparent, causing variance at the higher ratio measurements. Other causes of discrepancy may be due to non-linear binding characteristics of target to probe. Currently we can detect up to 300 fold ratio changes with accuracy.

6. Data Mining and Statistical Analysis.

All data from each experiment can be downloaded into a relational database such as FileMaker Pro (Claris) and further parsed for comparing data across experiments as well as for extracting data from individual array hybridizations. It is obvious that large-scale, high-throughput experimental methods require information processing coupled to a variety of analysis tools. Software tools such as ArrayDB (19) can also be used to integrate information from many Internet sources, such as NCBI Entrez, UniGene, and KEGG databases, with experimental gene expression data. Hierarchical clustering of biological samples and genes (14; 20; 21) is a commonly applied mathematical strategy to organize gene expression data. Algorithms such as multidimensional scaling are proving to be an informative way to visualize expression profiles (14). More complex data analysis systems (22-25) are currently being devised for complex clustering of data, a description of this is beyond the scope of this review.

NOTES

1. There currently exist in the literature a confusing interchangeable nomenclature system for referring to hybridization partners termed "probes" and "targets." For the purpose of this chapter we refer to the tethered DNA (of known identity) on the microarray slide as the probe, and the fluorescently labeled cDNA (synthesized from unknown mRNA messages) as the hybridization targets.
2. Use extreme care to avoid cross-contamination. Cross-contamination will be evident when PCR products are gel electrophoresed and present multiple bands. Contaminated clones will require re-streaking and sequencing.
3. It is recommended to make a slight excess of PCR reaction mix than what is actually required.
4. Take care to remove air bubbles and ensure proper mixing of reaction mix.
5. We use the FluoReporter Blue Fluorometric dsDNA kit, Dynex Microfluor plates, lambda HindIII fragments for standards, and a PerkinElmer Luminescence Spectrometer LS50B.
6. Ensure proper mixing of the PCR solution with the precipitation mix. Failure to do so will decrease DNA yield concentration.
7. Use rubber pads between the stacked plates to prevent cracking and breakage.
8. Heat and cool plates slowly to prevent condensation on the sealer and upper rim of the well.
9. It is important to wear powder-free gloves at all times and avoid contact with detergents or other compounds that may cause background fluorescence.
10. It is important to remove all traces of cleaning solution. Failure to do so will hinder poly-L-lysine coating reaction and will adversely affect microarray results (low intensity spots, high background).
11. Aged slides will be very hydrophobic (water drops leave no trail when they move across the surface).
12. A new slide should be marked for use as a template to line up the coverslip when putting together the microarray experiment, as the printed spots will not be visible after the slide blocking procedure.

13. The reaction solution must be prepared in completely dry containers. All glassware, stir bars, and graduated cylinders should be dedicated to slide blocking and should not be cleaned with detergents, which can adversely affect this reaction.
14. A number of groups have found that rapid or slow hydration of the DNA on the slide after printing, followed by a quick drying step improves DNA distribution or signal strength. This has not been observed for materials prepared by our procedure, so it is routinely omitted.
15. When water is added, the anhydride will begin to rapidly decompose, so add the mix to the slides very quickly. It is helpful to dispense the sodium borate buffer from a pre-aliquoted 50 ml conical tube.
16. Cover dish with aluminum foil to reduce evaporation and simultaneously boil ddH2O in microwave to replenish evaporative loss.
17. High quality RNA is crucial to the success of a microarray experiment. It is also possible to use 2 to 4 µg of poly(A)-purified mRNA in the target synthesis reaction. Smaller quantities of RNA may be used in conjunction with RNA amplification techniques, which are currently under development.
18. Cell lines should be harvested under consistent conditions and lysed rapidly. It is recommended that you sonicate the very viscous lysate with several 5-second bursts to disrupt the genomic DNA before applying to the RNeasy column. We use the micro probe (conical titanium probe 1.8 mm diameter tip) at a setting 5, dissipated power approximately 5-10 watts. When extracting RNA from tissue add the frozen sample directly to the Trizol without thawing with immediate homogenization.
19. Factor in an approximately 10% loss of RNA from each Trizol purification round.
20. Over-drying may make resuspension of RNA difficult and may adversely affect results.
21. Do not concentrate to dryness, as the sample may be lost or difficult to recover. Proper volume is when the filter is partially dry upon visual inspection.
22. The flow-through may be saved at this step for HPLC recovery of unincorporated fluorophores.
23. Appropriate competitor DNA should be used for microarrays of clones from other organisms, such as mouse.

24. For the best results, apply pooled targets to the center of the coverslip and then, using the template slide as a guide (see protocol 1.7), place inverted microarray slide from above.
25. Use paper towels and vacuum suction to completely dry outer surface of chamber.
26. Take care that the coverslip does not scratch the microarray surface.

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